Universidad Carlos III de Madrid

Bachelor Thesis

Confocal Microendoscopy

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Abstract

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Confocal Microendoscopy

by Isabel Martín Civiac

Confocal microendocopy has been pushed by the improvements in fiber optic technology and the miniaturization of optics and mechanics into the clinical domain. This new imaging technology provides the possibility of analyzing, at the microscopic level, cellular and sub-cellular features in vivo thanks to its high resolution. Together with a wide field fluorescence module, it will be used in animal models to study the performance of potential fluorescence contrast agents in the NIRF. More specific, the first application of our endoscope will be to study intestinal mucosa tissue using Acriflavine as dye in animals which have several tissue alterations, in especial colorectal cancer. This system has been designed to detect visible morphological changes in the mucosa structure that are characteristic from many diseases and conditions. We present an imaging system which will be accessible to everyone due to its reasonable price and flexibility of use.

Acknowledgements

Eternamente agradecida a todas las personas que me han acompañado en este corto pero gran viaje.

En primer lugar a mi familia. Gracias por darme todo, por haberme educado en los valores sobre los que hoy sostengo mi vida. Gracias papá y mamá por vuestro apoyo incondicional y por ser mi inspiración. A mi hermana, por dejarme en el camino un gran ejemplo a seguir.

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He sentido todo vuestro cariño desde muy cerca, sin vosotros no hubiera sido posible llegar hasta aquí.

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Chapter 1

Introduction

1.1 Gastrointestinal tract

The human gastrointestinal tract is an organ system where the digestion, absorption of nutrients and expulsion of wastes take place. It consists of two parts; the upper and lower tract. The buccal cavity, pharynx, esophagus, stomach and duodenum form part of the upper gastrointestinal tract whereas the lower gastrointestinal part is formed by most of the small intestine and all the large intestine [1].

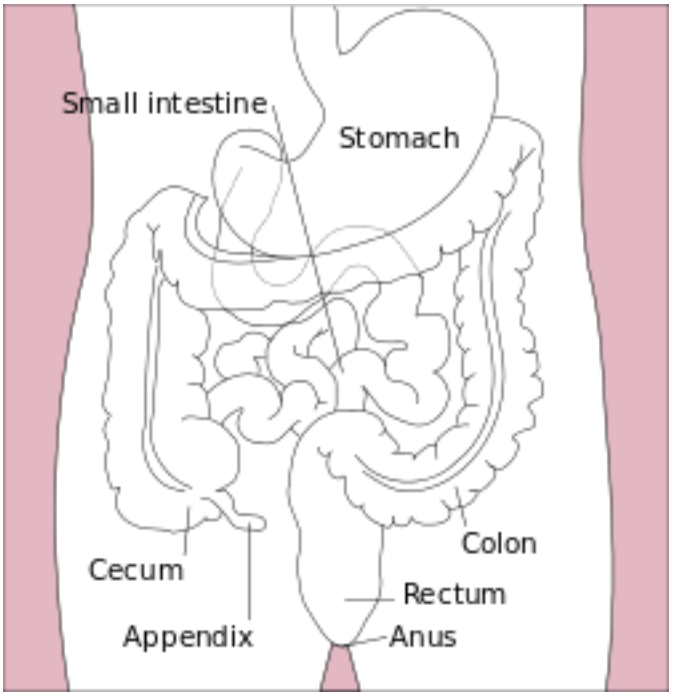

FIGURE 1.1: The human gastrointestinal tract (From Wikiwand).

In biology, the gastrointestinal tract is of vital importance and, therefore, is very interesting to study. There are many conditions and diseases that affect the gastrointestinal system such as Chron's disease, ulcerative colitis, diverticular disease or different cancer types. Nowadays, several techniques are been used to diagnose these diseases. Depending on where and on which structure the physician is interested in, radioopaque dyes, endoscopy or even abdominal x-ray can be done.

In the first technique a radiodense contrast media is used to track the path of the gastrointestinal tract in case there is any abnormality. Usually the preferred contrast agent is barium, which is swallowed by the patient. The ingested barium is followed by taking x-ray images of the regions of interest.

FIGURE 1.2: Barium swallow fluoroscopic image (From Wikipedia).

The use of abdominal X-ray as a diagnostic technique is not so usual. The advantages that it presents is that it can be performed easily in case of an emergency. In most of the cases it is used as a complementary technique which helps in the diagnostic of some abdominal conditions such as obstructions, malrotations or volvulus [2]. Usually, abdominal x-ray is not the first line of investigation to confirm the diagnostic, ultrasound imaging is used instead.

FIGURE 1.3: Abdominal X-Ray (From Wikipedia).

1.2 Endoscopy

Endoscopy is a medical procedure that is done using an instrument called endoscope, which is inserted into the body to observe and operate on the different organs, cavities or vessels [3]. The first attempt to look the human body through a tube was made by Philip Bozzini in 1805. He wanted to observe the urinary tract, pharynx and rectum but he did not succeed. It was not until 1853 when Antoine Jean Desormeaux developed an endoscope to observe the gastrointestinal tract. However, the first doctor who took a look at the living human body through an endoscope was Dr. Adolph Kussmaul from Germany in 1868 [4]. The examination of an endoscope is different from some imaging techniques such as X-ray or CT in which the images are taken of inside the body without the necessity of introducing any instrument or device inside the patient. Usually, an endoscopy carries the neccesity of performing a biopsy by means of histological techniques.

The endoscope consists on a long flexible tube with a light source which allows the cavity to be observable and a video camera at one end. However, it is important to point out that not all the endoscopes need a camera at its end since the images can be visualized directly by the eyes or transmited to an external device. In addition, it may have an additional channel in case that another medical instrument needs to be introduced.

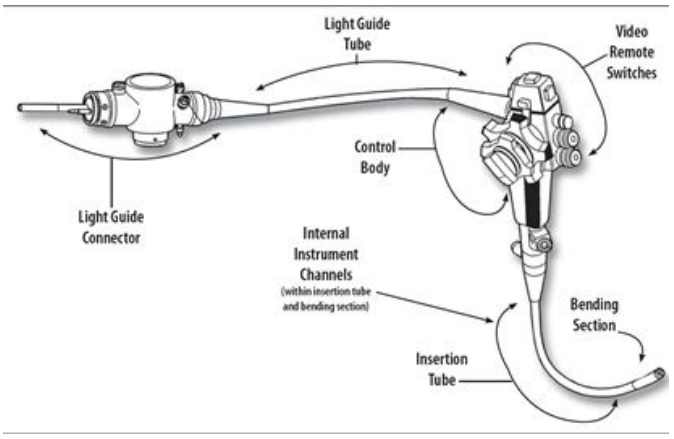

FIGURE 1.4: Typical Endoscope (From Educational Dimensions).

1.2.1 Confocal Endomicroscopy

Confocal endomicroscopy (CE) is a new modality that allows to obtain high resolution images of the living tissue which means that it offers important advantages over conventional microscopic modality [5–7]. This technique probably constitues the most important improvement in the diagnostics nowadays since it integrates both macroscopic and microscopic vision in real time [8].

The basis of the CE consists in obtaining an optical section from a thick biological tissue using fluorescence markers which are able to identify specific cellular receptors [9]. This technique is based on illuminating the mucosa tissue with a laser, which is absorbed by a fluorescent agent, being later the reflected light collected. The laser is focused at a specific depth and then the reflected light is redirected towards a detection system, excluding the reflected signal coming from out-of-focus beams. Therefore, the principle of the confocal endomicroscopy is based on eliminating the fluorescent or reflected signal not coming from the focused area.

It was first termed "confocal" in 1955 by Marvin Minsky since the condenser and objective lens had the same focal point [10].

Later, the traditional pinhole used in microscopy was switched to single mode fiber which has been proved to maintain the optical sectioning features of a microscope system better than a traditional system (Figure 1.6). A very thin UV laser arrives at an specific point in the tissue causing fluorescence. This signal is recieved by a selective filter preventing the pass for the rest of signals. Using an optic fiber, the complexity of the system is reduced and it allows the automatic alignment of the two appertures [10].

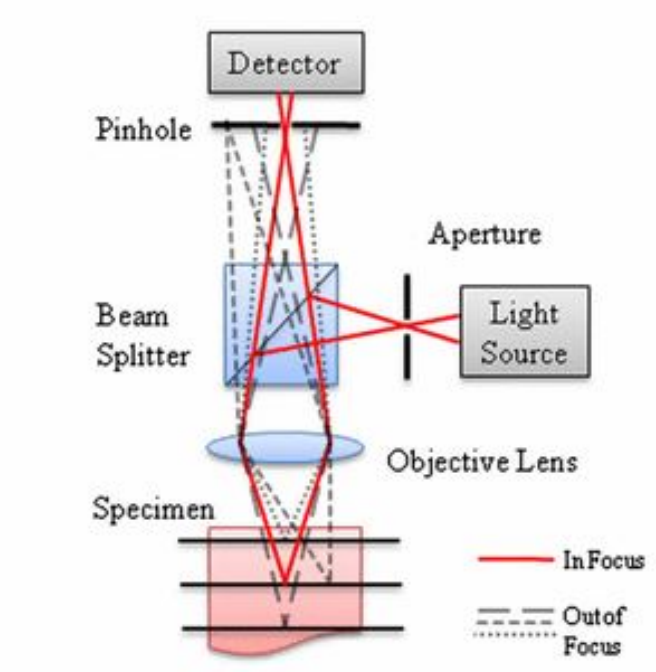

FIGURE 1.5: Schematic draw for confocal arquitecture showing rays coming from the focal spot (solid lines) and from out of focus regions. Out of focus rays are rejected by the confocal pinhole From Jabbour, J.M., et al., Confocal Endomicroscopy: Instrumentation and Medical Applications. Annals of biomedical engineering, 2012: p. 1–20; with permission)

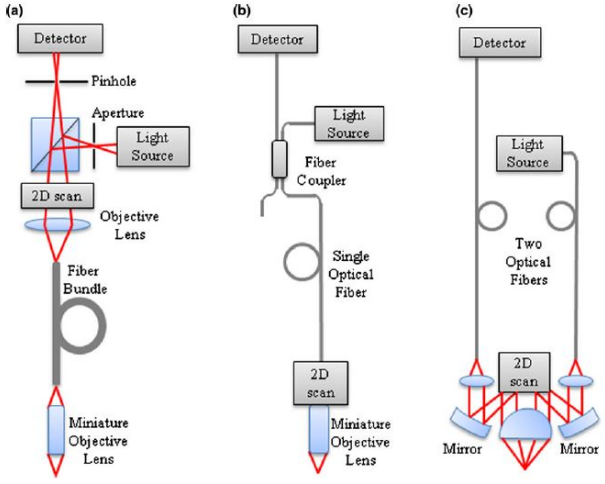

FIGURE 1.6: Configurations of the fiber confocal microendoscopes. (a) Proximally scanned endomicroscope using a fiber bundle for illumination and detection. (b) Distally scanned endomicroscope using a single optical fiber for illumination and detection. (c) Distally scanned endomicroscope using two optical fibers, one for illumination and one for detection. From Jabbour, J.M., et al., Confocal Endomicroscopy: Instrumentation and Medical Applications. Annals of biomedical engineering, 2012: p. 1–20; with permission)

1.3 Optical Fiber Technology

Fluorescence imaging techniques based on optical fiber constitute a major advance nowadays in medicine. They have hopeful applications in clinical studies [11]. There is a wide range of optical fibers; multi-mode, Single-mode, Bundle, GRIN, double-clad.... They are widely used in scanning microscopy and they work in different ways. For instance, fiber bundles deliver light to tissues and receive it from the same tissue to transmit the signal to a photodetector whereas double-cad illuminate the sample through some fibers and transmit the signal through others. The visualization through optic fibers allows to detect diseases and conditions that would be inaccessible by other means [12, 13].

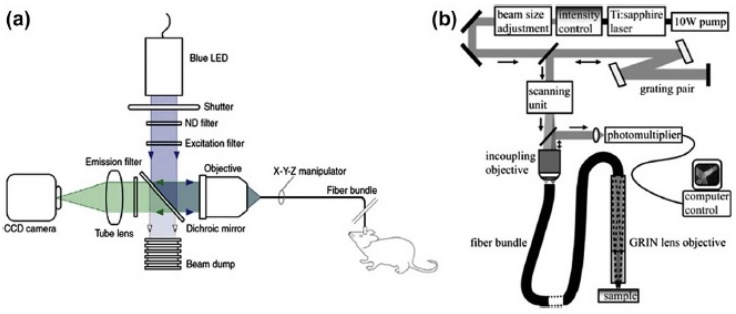

FIGURE 1.7: Schematics of fluorescence microendoscope with fiber bundle. (a) One-photon modality, an epifluorescence microscope attached to fiber optic system for in vivo imaging. (From Murayama, M. and M.E. Larkum, In vivo dendritic calcium imaging with a fiberoptic periscope system. Nature protocols, 2009. 4(10): p. 1551–1559; with permission) [89]. (b) Two-photon modality, Femtosecond laser pulses pass a pair of diffraction gratings for pulse compression before they are coupled into fiber bundle through a standard two-photon laser-scanning microscope. Fluorescent emission is detected through the fiber bundle and the in coupling objective by a photomultiplier tube. (From Göbel, W., et al., Miniaturized two-photon microscope based on a flexible coherent fiber bundle and a gradient-index lens objective. Optics letters, 2004. 29(21): p. 2521– 2523; with permission)

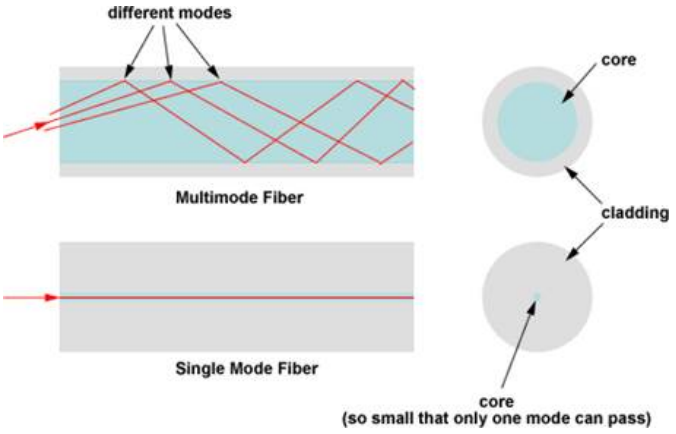

FIGURE 1.8: Schematics of a single mode fiber and multimodal fiber. (From Optical Fiber 4 Sale)

However, this modality has some disadvantages such as that the penetration depth is very little, usually less tan 100 micrometers from the tissue surface. Also, fiber bundles have the disadvantage of the pixelation artifact caused by the non-imaging spaces between the fiber cores.

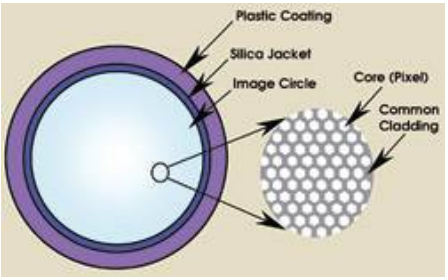

FIGURE 1.9: Fiber Bundle Cross section (From Walker R. and Bessant N., Customization of optical fiber leads to new applications in monitoring, manufacturing and research. Photonics Spectra)

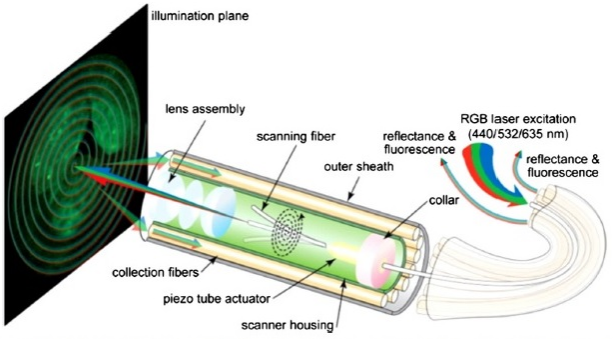

FIGURE 1.10: Functional diagram of the Multispectral SFE with the scanning illumination fiber moving in the spiral scan pattern. RGB laser excitation (440, 532 and 635 nm) is delivered into a single-mode fiber that is scanned in a spiral pattern by a piezo tube actuator and focused onto the tissue (illumination plane) by a lens assembly. Fluorescence is collected by a ring of 12 collection fibers mounted around the periphery of the scanner housing, protected by an outer sheath. (From Miller, S.J., et al., Targeted detection of murine colonic dysplasia in vivo with flexible multispectral scanning fiber endoscopy. Journal of biomedical optics, 2012. 17(2): p. 021103-1–021103-11; with permisson)

A typical fluorescence microendoscope consists of a a fiber bundle, light source, various lenses and a CCD camera where the excitation light comes from lasers, lamps or LEDs. With a confocal microendoscope enhacement of lateral and axial resolution can be obtained. In addition, it has the advantage of being able to reject the out-of-focus background using a confocal pinhole [10].

1.4 Fluorescence Diagnostics

Fluorescence is a specific type of luminescence that characterizes the substances which are able to absorb energy in form of electromagnetic waves and, then, re-emit part of that energy as electromagnetic radiation with a different wavelength [14].

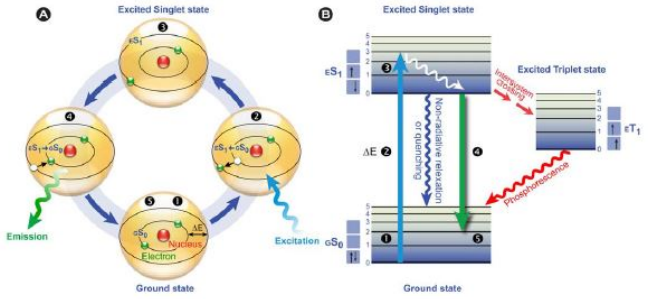

FIGURE 1.11: Fluorescence principle. (a) Schematics of the fluorescence phenomenon based on the classical Bohr model. (b) Jabłoński diagram: Upon photon absorption, a ground state GS0 electron (electronic singlet) is promoted to a higher and excited state, relaxes quickly to a lower vibrational excited state (white line) and thereby looses energy. When returning to the ground state, it dissipates the remaining energy by emitting a photon with a longer wavelength, i.e., fluorescence emission (From Drummen G.P.C, Fluoresnct Probes and Fluorescence (Microscopy) Techniques- Illuminating Biological and Biomedical Research. Molecules 2012, 17, 14067-14090)

Its development had an important effect on biomedical research since it allowed scientists not only to observe normal physiological processes which could be seen with other techniques but also to detect and analyze several signals and to track them in real time. Therefore, fluorescence has become a good choice as a diagnostic method. With the help of fluorescence, it is possible to access organs and cavities without the need of going through a surgery.

Molecules that show fluorescence are usually named fluorophores or fluorochromes and there is one specific fluorescent compound that has a major impact in life scince allowing to gain new perception in all its disciplines and this molecule is named Green Fluorescent Protein [14]. These molecules usually show 3 stages in fluorescence phenomenon. The first stage is excitation where a photon is emitted using a laser or an external light source with certain energy. Then, the fluorophore absorbs that energy and, as a consequence, it reaches an excited state that lasts between 1 and 10 nanoseconds.

During the second stage, the molecule suffers conformational changes and the energy is almost dissipated, yielding a relaxed singlet excited state where the fluorescence emission originates. However, not all the excited molecules return to the original state by fluorescence emission.

In the third and last stage, the fluorochrome returns to the ground state giving fluorescence [8, 14, 15].

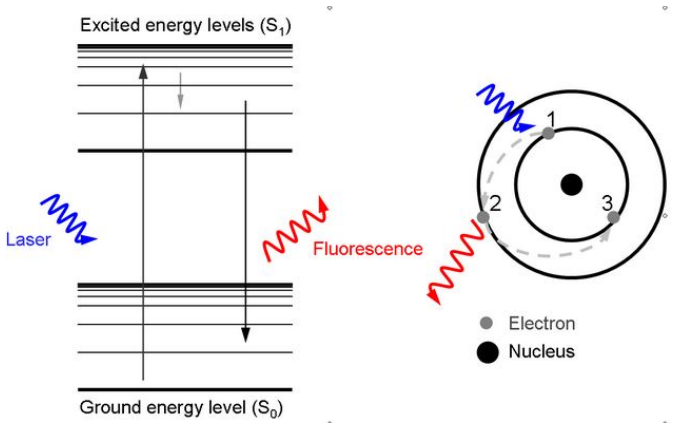

FIGURE 1.12: Fluorescence phenomenon using an energy level diagram (From Wikimedia)

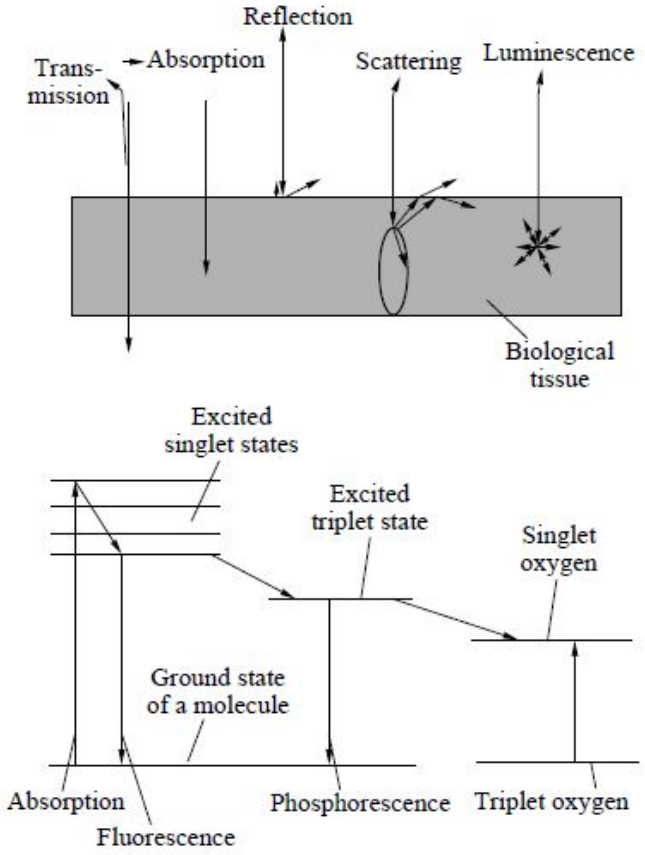

FIGURE 1.13: Scheme of the interaction between light and biological tissues (From Loschenov V.B., Konov V. I. and Prokhorov A. M., Photodynamic Therapy and Fluorescence Diagnostics. Laser Physics, Vol. 10, No. 6, 2000, pp.1188-1207.)

Fluorescence is a promising technique that still has much to offer since it is a relatively new modality. It has already been used in many imaging modalities and assays but new developments are yet to come in both fluorochrome and fluorescence imaging.

Chapter 2

Objectives and Motivation

2.1 Endomicroscopy module

The path of the medicine is characterized for its constant and continuous development. This progress has the objective of treating the human diseases and conditions in a more specific way, with higher efficacy and, more importantly, with a cheaper technology. As it is widely known, it has been greatly challeging to integrate a confocal microendoscope in the distal end of an endoscope but the results are very satisfactory [16]. New perspectives are being created because of the ability of seeing at cellular and subcellular levels in all kind of tissues.

The main objective is to create an endomicroscopy module which has an acquisition rate enough to obtain in vivo and in situ images of samples in movement with high resolution. For this purpose, we are going to test two different configurations in this work: Confocal endomicroscopy and Real-time endomicroscopy.

2.1.1 Confocal Scanning Endomicroscopy

The use of endoscopy has evolved in the last decade to detect gastrointestinal diseases and conditions [17]. There are many different endoscopy techniques where each one has its own applications. The early diagnosis of diseases such as ulcerative colitis, Chron's disease or colorectal cancer is crucial and with confocal endoscopy is possible. With the early visaluzation of alterations in the gastrointestinal mucosa, those inflammatory processes could be treated from the very first stage and the severity of the disease would be reduced.

Taking into consideration the state of the art of this kind of systems as well as of fluorescence microscopy, we have decided to design a new generation of endoscopes that integrates both confocal fluorescence endomicroscopy which enables high resolution imaging using different contrast agents together with a near-infrared fluorescence (NIRF) which enables specific imaging of disease's markers.

With this multimodal imaging system, we will be able to detect areas in which the tissue is altered with the wide field module and once we have detected that possible alteration, use the endomicroscopy module to obtain an image with subcellular resolution. It is also worth mentioning that the position of the fiber bundle used in endomicroscopy will be seen and controlled by the wide field module. Thus, we will know which area we are observing with high accuracy.

Moreover, taking advantage of the lack of cheap systems, the objective of this project is the creation of a confocal endomicroscopy system which allows an accurate, cost-effective and fast diagnostic. Also, the lack of specific markers which allows to detect early lesions is what moved us forward into this project.

2.1.2 Real-time Endomicroscope

In addition, a real-time endomicroscope has been developed. The motivation to design a new system comes from the need of having real-time images which opens a new and promising future for the medicine. With this wide-field real-time endomicroscope the requirement of taking samples through a traditional biopsy would be reduced in some cases. Thanks to the possibily of visualizing in vivo histology, "localized" biopsys could be made instead of random ones which will provide more accuracy.

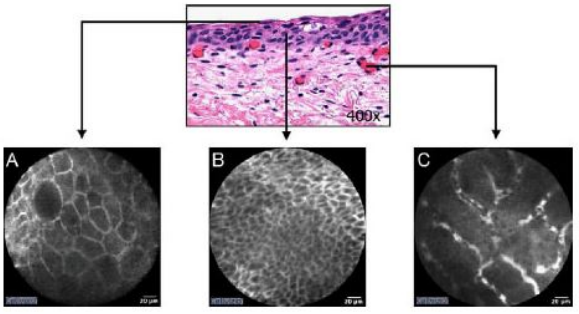

FIGURE 2.1: Comparison between images seen by traditional histology and by confocal endomicroscopy (From Oliva. J, Marco. A, Pelegrí. J, Rioja. C, Beyond the photodynamic diagnosis: Searching for excellence in the diagnosis of non-muscle-invasive bladder cancer)

Chapter 3

Materials and Methods

In this work, we have developed two different systems: Confocal and Real-time endomicroscopy. The confocal module offers a great resolution which is very important in this field. However, this high resolution compromises the acquisition rate, which in this case is not as fast as needed for in vivo imaging. This is why we also present in this study the Real-time endomicroscopy module which offers, in contrast, images in real time with enough good resolution.

Based on the confocal microscopy principle, we have designed our confocal system. The main difference with respect to the original mounting is that a fiber bundle is used as the predominant element.

3.1 System Assembly

3.1.1 Confocal endomicroscopy module

In this confocal module only one fiber of the fiber bundle is illuminated at a time and the galvanometer mirrors are used to scan the fiber bundle, so that each fiber is illuminated independently. Then, the fluorescent signal that comes from the sample through the fiber bundle is collected, in a way that all the fibers gather some light, even though this light is more intense in the fiber that has been illuminated. In this kind of systems a pinhole is needed which is placed before the avalanche photodiode in order to block the fluorescence originated in the fibers which have not been illuminated. In this way, only the signal that comes from the illuminated light arrives at the sensor. Thus, improving the resolution of the image but losing acquisition rate.

For the assembly of the confocal microendoscope the following components were needed.

3.1.1.1 Fiber Bundle

Fujikura has been chosen as manufacturer since they provide with a wide range of silica-based coherent fibers for medical purposes. Its fibers have great properties such as low transmission loss and, also, high mechanical durability which is very important for this project since it can be used in other medical application.

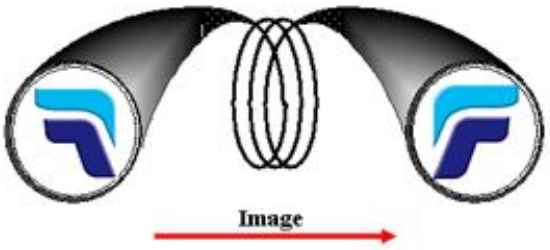

FIGURE 3.1: Fiber bundle (From Fujikura Europe Ltd)

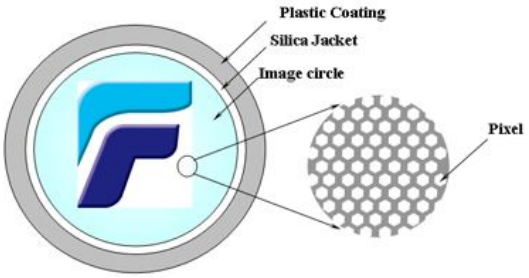

FIGURE 3.2: Cross Section Structure of a Fiber Bundle (From Fujikura Europe Ltd)

We have chosen FIGH series N-type since they offer super-thin fibers providing great image quality with very high-resolution in small outer diameters. Also, it offers great contrast and color with no defects. Therefore, this kind of fibers are ideal for minimally non-invasive endoscopes. The fiber chosen for this project has 30.000 fibers and it has a dimeter of 1mm. The resolution will be determined by the core-to-core distance which in this case is 4.5 micrometers whereas the field of view (FOV) will be determined by the diameter of the fiber. By illuminating only fiber cores and not the spaces between them, the contrast and optical sectioning will be improved.

3.1.1.2 Laser

A laser diode module is needed to illuminate the sample. A CPS 520 from Thorlabs was chosen. It has a wavelength of 520nm and the beam sheap is of 4.6mm x 1.7mm.

FIGURE 3.3: Collimated Laser Diode Module CPS 520, Thorlabs Inc, Delaware, USA

3.1.1.3 Galvanometer Mirrors

Using 2D Galvanometer mirrors, the sample is ulluminated with the help of the laser diode module to scan the fiber bundle which has 30.000 fibers. These high-speed Scanning mirrors are specially good for endoscopy applications where a fast scanning is needed.

FIGURE 3.4: Large Beam Diameter Dual-axis Scanning Galvo System. GVS012, Thorlabs Inc, Delaware, USA.

3.1.1.4 Photodiode

The Silicon Avalanche Photodectector from Thorlabs Inc provides high sensitivity and it reduces noise in comparison with the standard detectors. The photodiode is used in a way that it collects the fluorescence coming through the fiber bundle which is in charge of carrying the illumination until the sample.

3.1.1.5 Lenses and Objective

For our project, two different lenses have been used 3.5. The first one is a scan lens LSM02 from Thorlabs Inc whose main property is that it minimizes back reflections. Also, a relay lens and an objective are needed. 3.6 3.7. All these 3 components are

FIGURE 3.5: Temperature-Compensated Silicon Avalanche Photodetector. APD130A2,Thorlabs Inc, Delaware, USA.

placed behind the galvanometer mirrors and is used to focus the light coming from the laser in each single fiber of the fiber bundle independently. The two lenses work as in a traditional microscope, they are fixed in a position which focus best. We have carefully chosen that position to optimize its performance. However, this position can be changed.

FIGURE 3.6: Scan Lens LSM02, Thorlabs Inc, Delaware, USA

FIGURE 3.7: Relay Lens, Achromatic Doublets AC254-030, Thorlabs Inc, Delaware, USA

FIGURE 3.8: Microscope Objective Lens Plan Apochromat 10X, Motic, Weztlar, Germany

3.1.1.6 Dichroic Mirror and Emission filter

The dichroic mirror and the emission filter are used to separate different wavelengths so that it will only let pass ligth at the wavelentgh we are interested in, the one corresponding to the fluorescence. In this way, only fluorescent light will come to the photodiode.

FIGURE 3.9: 45º Reflective Dichroic Color Filter. Edmund optics, Newport, NJ, USA

3.1.1.7 Pinhole

The pinhole is placed between the two lenses in order to avoid optical aberrations. This instrument will only allow the pass of light coming from an illumated fiber, blocking the pass of non-illuminated-fiber light and, therefore, increasing the resolution of the resulting image. Finally, the light exiting the precision pinhole is collected in the photodiode. A pinhole P25S from Thorlabs Inc is used in this work.

3.1.1.8 Endoscope

In this study, an Examination Sheath endoscope has been used. We have chosen the model STZ 61029D from the company Karl Storz, Germany. It has a working length of

7 cm with Obtuator 61029 DO. Also, it has a channel diameter of 1 mm, so the fiber bundle fits inside perfectly meaning that both microscopic and macroscopic view can be seen at the same time. Since both images can be acquired, we know the position of the fiber at any time with the help of the wide field system.

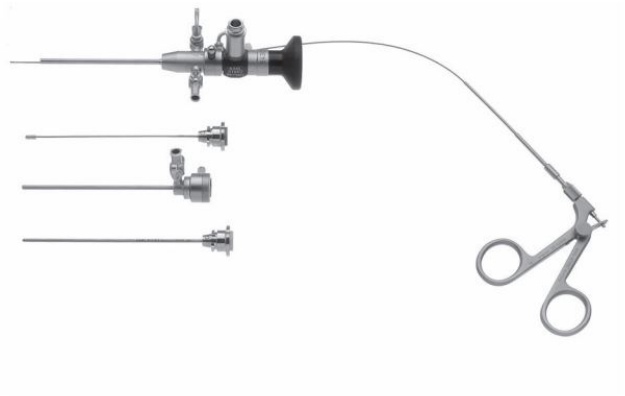

FIGURE 3.10: Mainz COLOVIEW System.

Finally, all these optical components were assembled in a 30mm cage-assembly system.

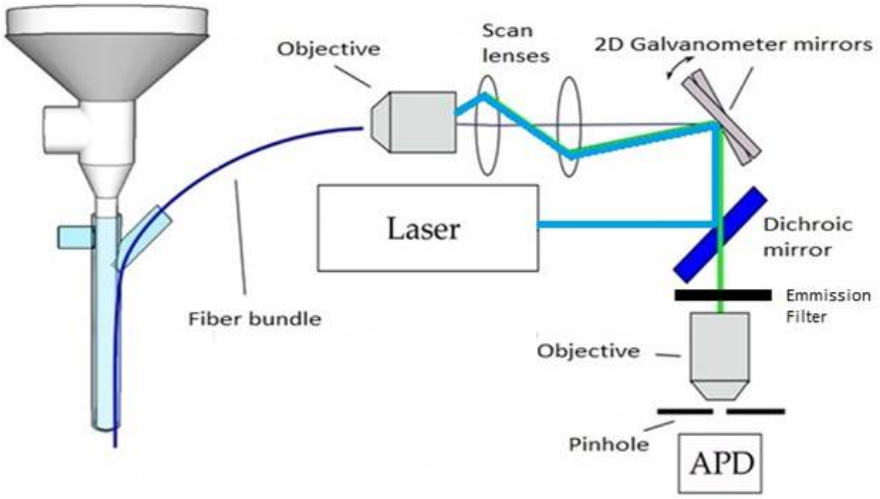

FIGURE 3.11: System assembly of the Confocal endomicroscopy module.

3.1.2 Real-time Endomicroscopy module

For the assembly of the real-time endomicroscope, we reused some components from the setting up of the confocal module such as the lenses and objective, fiber bundle, dichroic mirror, emission filter and the laser. However, a new component is added to this mounting: a high-resolution CMOS camera. We have chosen DCC1545M which is a monochrome sensor, it has a resolution of 1280x1024 pixeles and a frame rate of 25.

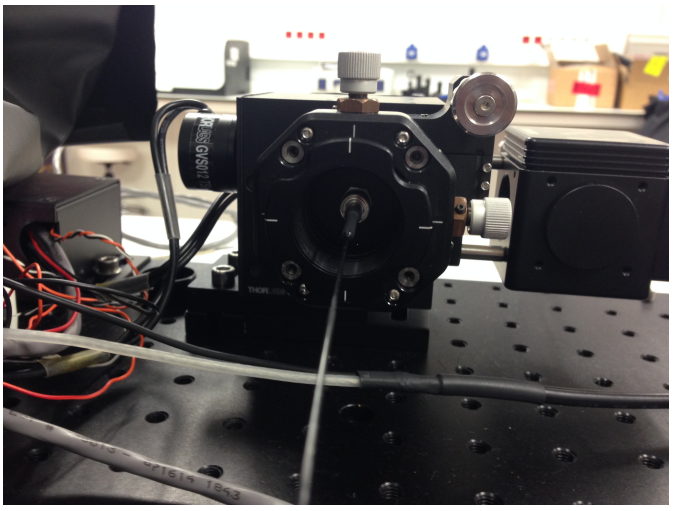

FIGURE 3.12: Real aspect of the confocal endomicroscopy module.

In this real-time module, the excited signal leaves the distal end of the fiber bundle in order to illuminate the sample and the emitted light is then collected and sent thorugh the objective, dichroic mirror and emission filter before forming images onto the CCD camera.

FIGURE 3.13: CMOS Camera DCC 1545M, Thorlabs Inc, Delaware, USA.

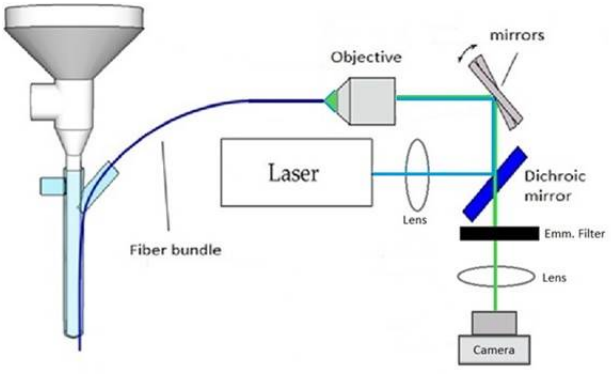

FIGURE 3.14: Scheme of the Real-time endomicroscopy module.

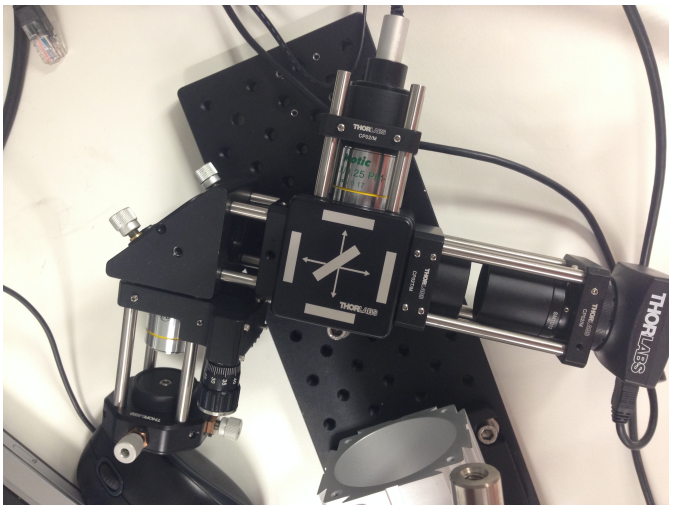

FIGURE 3.15: Final assembly of the Real-Time Endomicroscope Module

3.2 Software Development

In this work, we have used LabView (Laboratory Virtual Instrumentation Engineering Workbench). It is a visual programming language developed by National Instruments what makes it different from the rest of programming languages such as C, C++, Java, etc.

In the confocal endomicroscopy module, the main objective of the developed programme is to scan each fiber of the fiber bundle as fast as possible. The development of the software was a great challenge since the synchronization of the galvanometer mirrors with the photodiode was not easy. Moreover, the communication between the mirrors and the photodiode is established through a Data Acquisition Card (DAQ NI 6009, National Instruments) which is a limitation for the time of scanning. Our software allows to scan in three different ways depending on the user requirements: manual, slow and fast.

3.2.1 Slow Scan-Confocal Module

This is a very simple way of scanning. First of all, a matrix with all the x and y values that will be scanned is created in a separate SubVI which is called in the main file. The dimensions of the matrix are from 1 to Ny in y and from 1 to Nx in x and the interval between points has been chosen to be 0,01. We have decided the interval to be such small because the smaller it is, the more accurate the image will be. Once we have the two arrays, one for x and another for y, the programme takes one point from each array and enters the subVI called "Position". This function moves the galvo mirrors

to the desired x,y position and the photodiode collects a measurement. This process is repeated for each position and this is why it is a very slow method. Finally, once the scanning has ended, an intensity graph comes out as a result. The image obtained has good resolution.

The inputs of this scanning are Nx, Ny, offset x, offset y, dx and dy.

3.2.2 Fast Scan-Confocal Module

Fast Scan is the fastest way of scanning with our confocal endomicroscope. First, we have created 2 sawtooth functions, one for x and other for y, so that each galvo mirror moves independently. The X galvo mirror moves at a very high frequency while the Y galvo mirror moves much slower in order to let the X mirror to scan from one side to another. This action is repeated a predetermined number of times. In this way, while the mirrors are moving, the avalanche photodiode collects data. However, we need to know the exact position of the galvos each time the photodiode reads a measurement to construct an image later. For this reason, the DAQ reads from 3 different channels the x and y position and the intesity it gets at that point. The photodiode stops taking measurements when the mirrors do. Finally, once we have the array with all the collected data, the points are interpolated so that to obtain the intensity graph. This interpolation is needed because all the collected values are not in order.

Since the natural breathing movement of the animal can distort the image, the most important paramenter is the acquisition time. This is why the fast Scan method is more practical than the Slow Scan.

The inputs of this scanning method are: frequency, offset, phase, amplitude, sampling info, sample rate, samples per channel and samples to read.

3.2.3 Manual-Confocal Module

This function allows the user to place the mirrors in a specific position using a similar function as in the Slow Scan. Also, an intesity value can be obatined. It is the simplest method of scanning and this is why it is mostly used to calibrate the system.

3.2.4 Real-time Module

Seen that the acquisition time of the confocal endomicroscopy was high, we then decided to implement a new module using a CCD camera instead of the confocal assembly. For

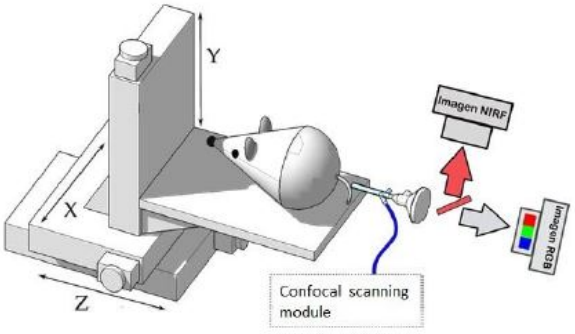

FIGURE 3.16: Set up of the multi-modular endomicroscopy in animal studies.

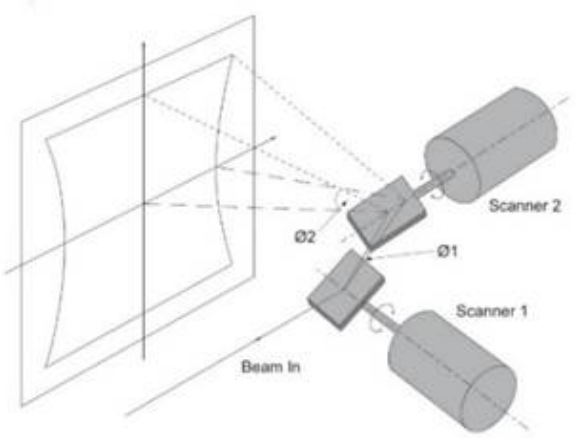

FIGURE 3.17: Scheme of the distortion in a system with two mirrors.

this purpose, we developed another programme in which the user can record a video or take an image and they are automatically saved in a specific folder. The advantage of this system is that we are seeing in real-time and the resolution of the video and images is good enough. The function of this method is much simpler since it does not have to scan any fiber. The video format is .avi and the user has to choose the value of the frames per second which, in this case, the camera we used allows a maximum of 30 fps.

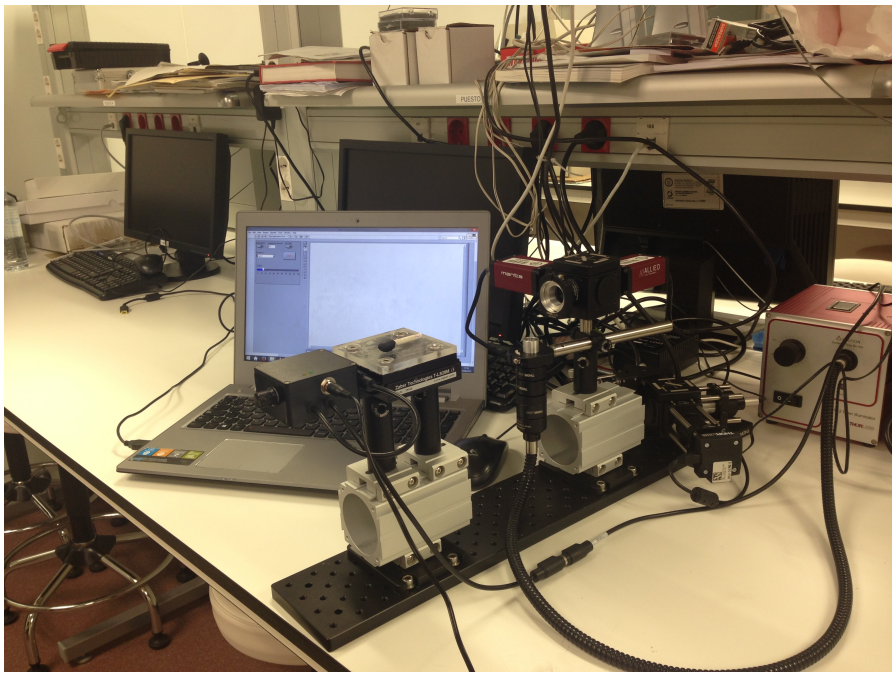

FIGURE 3.18: Aspect of the Real- Time Endomicroscopy Module.

Chapter 4

Results and Discussion

The other module that works together with the one we present in this work allows the observation of the desired area of a tissue through a 30fps video. Therefore, a possible lession previously detected with this macroscopic module is later deeply analyzed with the microscopic system which allows the examination at a cellular and subcellular level.

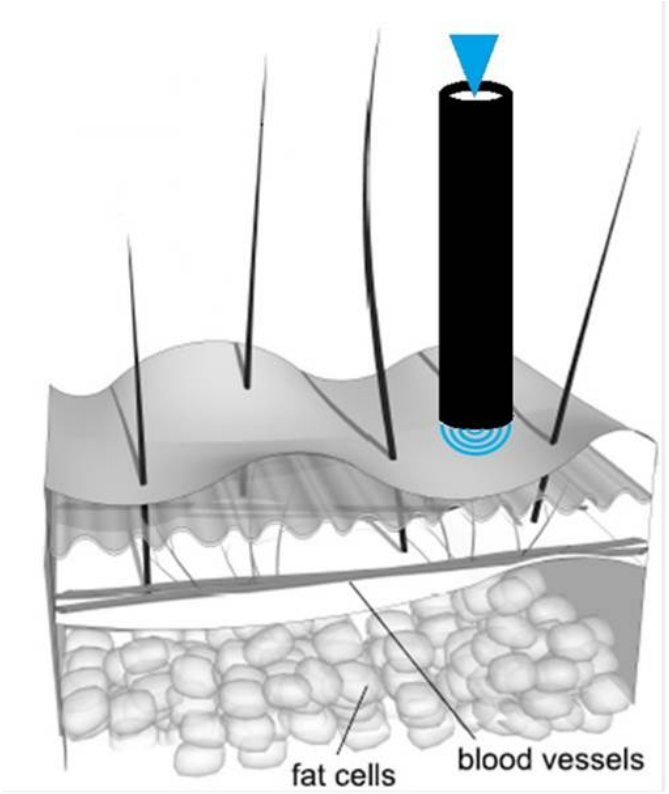

FIGURE 4.1: Scheme of the placing of the fiber in direct contact with the tissue.

The Slow and Fast Scan methods have been developed in order to obtain images with high-resolution and with the minimum acquisition time possible. The major challenge of

this project was to have a good field of view whithout compromising either the resolution nor the rate of acquisition. However, we take advantage of the wide field module for dealing with that problem since it allows the visualization and the monitoring of the fiber from its cameras. Thus, we could reduce our field of view.

One of the advantages of our system is that since the field of view is determined by the diameter of the fiber bundle and the one we use is of 1mm, we can see as many cells as fit in that space. Taking into account the average cell size, hundreds of cells can be observed in an image.

With the developed software we are able to scan an area in two different ways with the confocal endomicroscopy module. The first method consists on a slow scanning in which the galvanometer mirrors moves to a position and the photodiode takes a measurement. The main disadvantage of this function is that it is time-consuming, it can take up to 100s to scan 400x400 points with a resolution of 3 micrometers. However, the synchronization between the mirrors and the photodiode is good compared to the fast scanning process and the position of the mirrors is very accurate leading to a minimum error.

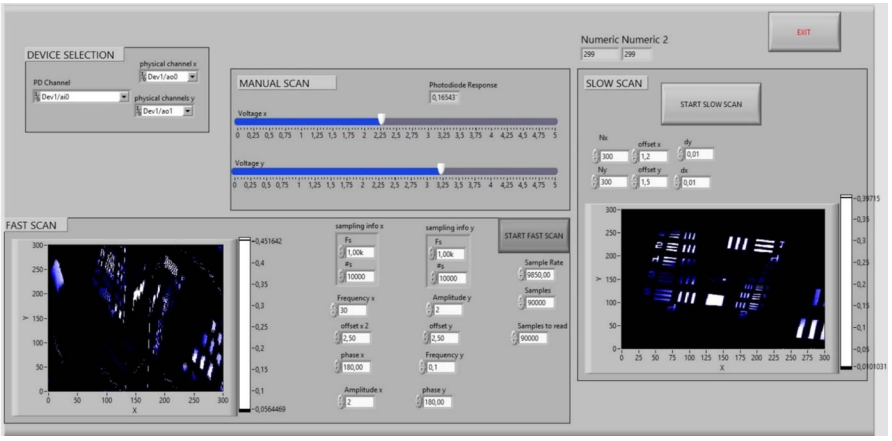

FIGURE 4.2: User-friendly interface of the confocal scanning with both slow and fast scan methods. Images taken of an USAF 1951 resolution test.

The other way of operation is with the fast scan method. In this case, the mirrors moves independently while the avalanche photodiode takes measurements at a certain rate. Also, each time the photodiode measures, we need to know in which position the galvanometer mirrors are in order to be able to construct the image later on. The Fast Scan constitues the most feasible way of scanning with the confocal assembly because the acquisition time is low and the images have a relatively good resolution. We have to take into account this parameters since this system will be used for in vivo studies and the natural breathing movement of the animals can disturb the images. The comparison between both methods regarding the field of view, the number of points scanned and the required time is seen in 4.1.

TABLE 4.1: Slow and Fast Scan Comparison.

Scanning Type	FOV	Resolution	Points	Time
Fast Scan	$1m^2$	3	400x400	6s
		4	300x300	5s
		8	150x150	3s
		12	100x100	2s
Slow Scan	$1m^2$	3	400x400	100s
		4	300x300	60s
		8	150x150	15s
		12	100x100	10s

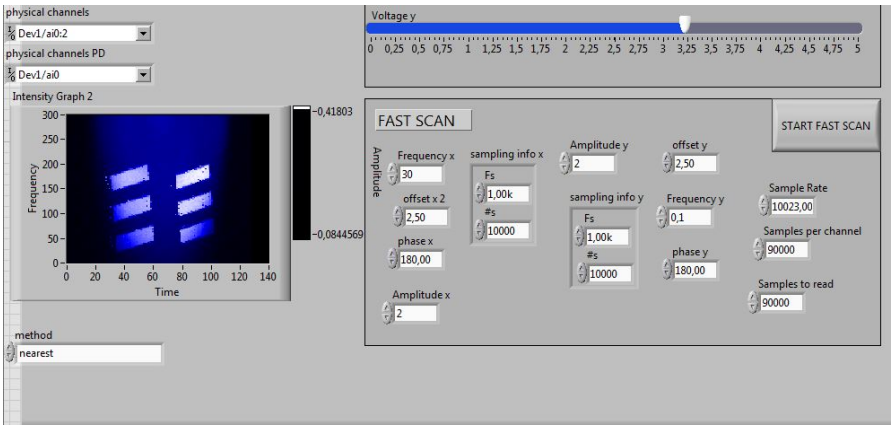

FIGURE 4.3: Image taken with the Fast Scan Method.

Because of the need of obtaning images in real-time so that the animal movement does not affect the images, we developed a different configuration. In this configuration, we made use of a CCD camera and the result was surprising. We can record video and take images with a good contrast and its simplicity makes it to be the best option when performing an endoscopy. It also allows different fluorescence channels.

We have used acriflavine as fluorosphore and the cellular morphology of the upper and lower gastrointestinal tract could be observed in detail. We analyzed 4 different parts of the GI tract of a rat (Figures 4.5, 4.6, 4.7, 4.8)

In all the following images the different structures can be visualized. In figures 4.4 and 4.5, several hundreds of nuclei can be observed thanks to the action of the Acriflavine. It is worth mentioning that the Acriflavine has a limited time of fluorescence so we tried to observe when the fluorescence was maximum in order to obtain better images. We also had to take into account that the concentration of acriflavine is not a trivial thing, it has to be carefully measured so that it is neither too much nor too little. For this purpose, we prepared different solutions of acriflavine with concentrations of 0.1, 0.09, 0.08 and 0.07.

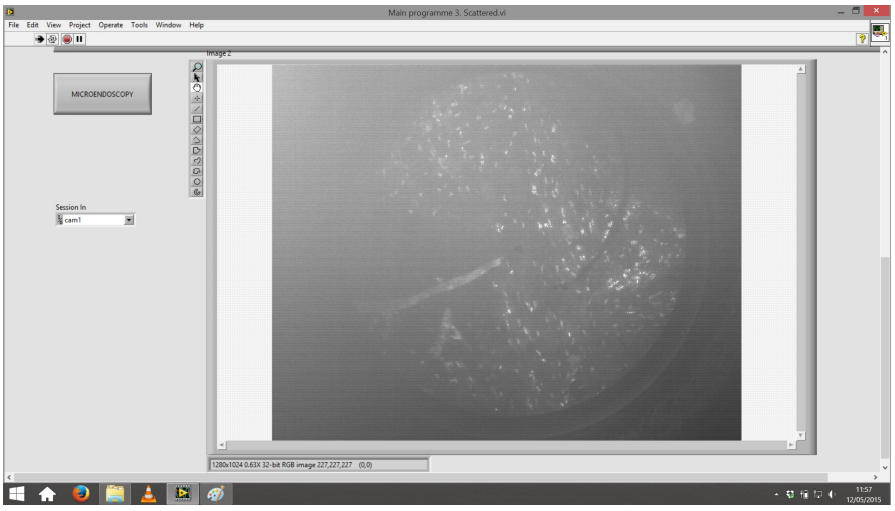

FIGURE 4.4: Interface of the Real-Time Endomicroscopy module examining the mucosae of the GI tract.

In figures 4.6 and 4.7, the plasmatic membrane can be also distinguised together with the nuclei in the middle of it. The resolution of these images are good and many structures can be observed.

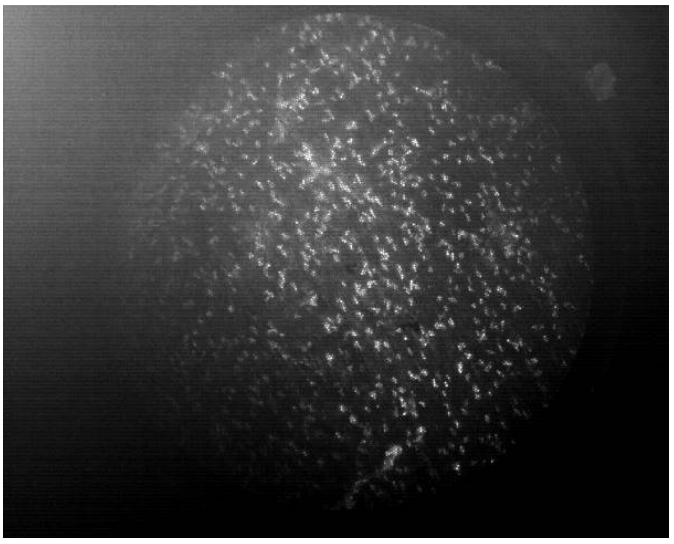

FIGURE 4.5: Exvivo image of an middle-upper area of a mouse's intestine using Acriflavine.

Also, we observed healthy human gingiva tissue (Figures 4.9, 4.10, 4.11) treated with acriflavine and the images obtained were good what strongly suggests that this real-time endomicroscopy module has a prominent future. As well as in the case of the other images, in theses ones the nuclei and, in some cases the membrane, can be crearly distiguised.

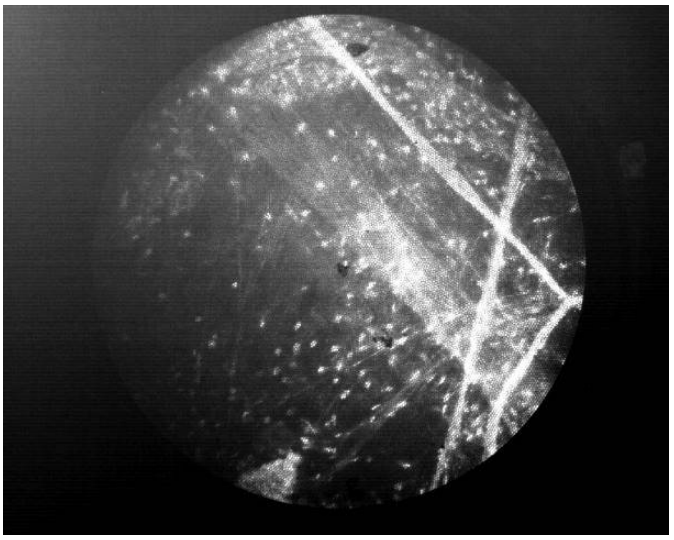

FIGURE 4.6: Exvivo image of an upper area of a mouse's intestine using Acriflavine.

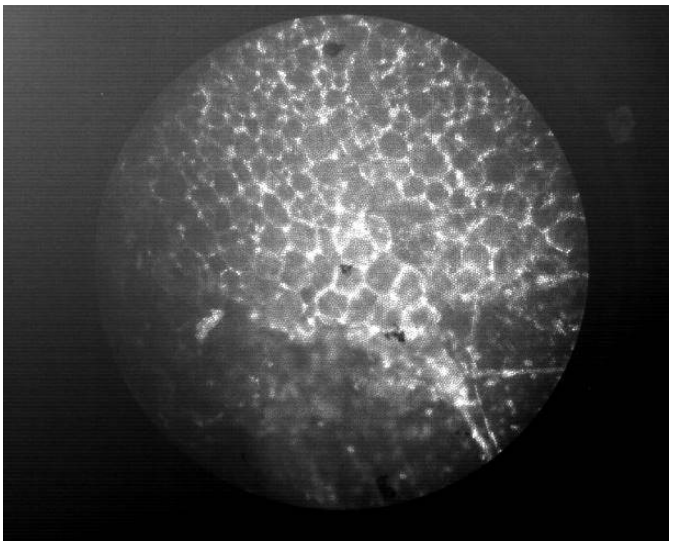

FIGURE 4.7: Exvivo image of a middle-lower area of a mouse's intestine using Acriflavine.

In contrast with the wide field module, our Real-Time endomicroscopy module allows to record a video with a resolution of a more or less 4 μm against the 1280x 1024 that offers the other system. The field of view is also remarkable since our fiber bundle offers 1mm FOV taking into account that both modules offer a video with 30fps.

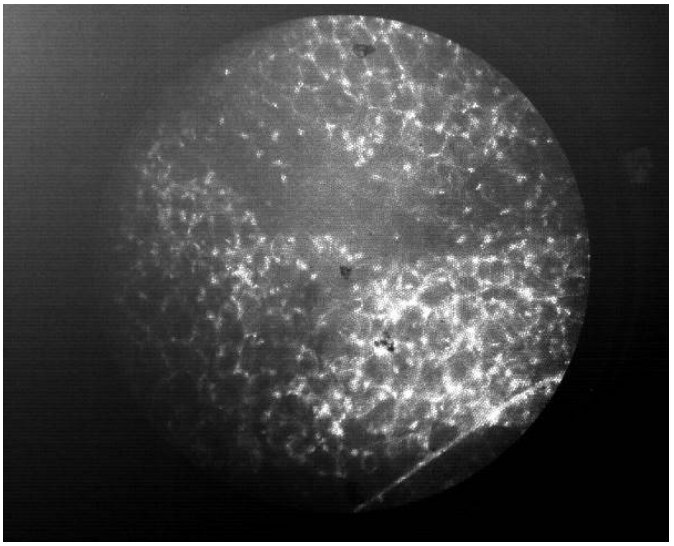

FIGURE 4.8: Exvivo image of a lower area of a mouse's intestine using Acriflavine.

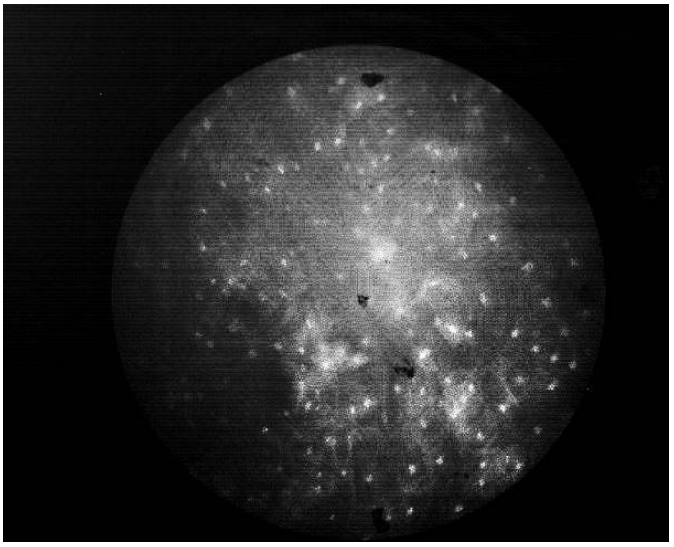

FIGURE 4.9: Exvivo image of healthy human gingiva with Acriflavine.

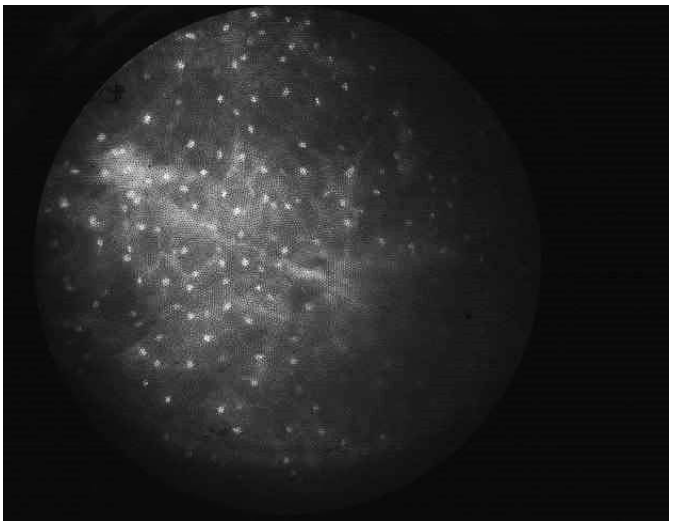

FIGURE 4.10: Exvivo image of healthy human gingiva with Acriflavine.

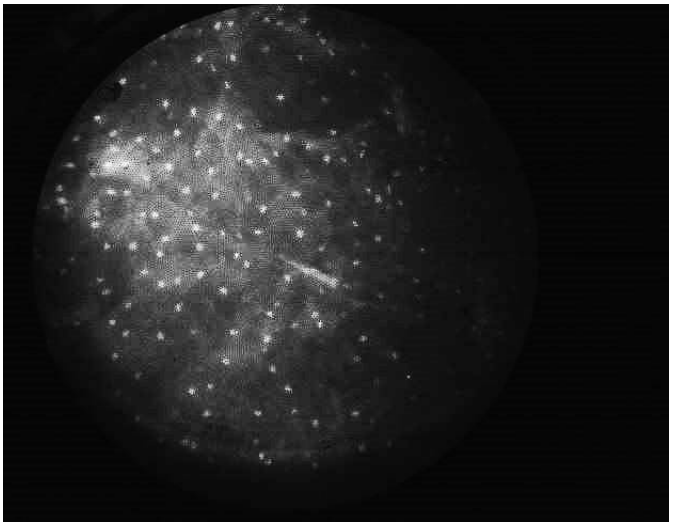

FIGURE 4.11: Exvivo image of healthy human gingiva with Acriflavine.

Chapter 5

Conclusion and Perspectives

5.1 Conclusion

A high-resolution confocal endomicroscopy system has been developed with a fast rate of acquisition and a wide field of view for its use in animal studies. This system will be used in the Department of Cell Biology and Immunology, in the Centro de Biología Molecular, in Madrid together with the wide field module which enables the combination of Near Infrarred Images and RGB. This multimodal endoscopy is capable of visualizing at both macroscopic and microscopic level. We here also present the software of the confocal and real-time endomicroscopy, a cheap and effective solution accessible to everyone thanks to its user-friendly interface.

Our system opens the possibility of having an early diagnosis of the diseases and conditions of the gastrointestinal tract such as Chron's disease, Ulcerative Colitis or Colorectal Cancer. With the obtained images earlier lessions at the mucosae structure can be detected. It will be used in murine models which have GI diseases for in vivo-imaging.

5.2 Perspectives

Some improvements can be done in order to optimize the system. The resolution is always an important parameter in this kind of systems so it could become better if a more powerful laser was used. The use of a better laser would lead to a reduction of the M^2 factor. Another critical parameter is the acquisition time and it could be reduced using another Data Aquisition Card (DAQ) with higher speed.

Additionally, since the morphology of the cells is crucial when diagnosting a disease, further developements in the software could be made. For instance, in the images we

obtain with our system there is a black space between the nuclei and the membrane. A new functionality could be added in which these black points were mapped in squares and, in this way, know the exact shape of the cell and the physicians would be able to give a faster and more accurate diagnostic.

Also, a new post processing software could be developed in order to do a quantitative analysis for the number of nuclei and the size of the cells. These parameters also account in the diagnostic of any biological process.

Finally, changing between one method and another could be done automatically so that there is no loss of time doing it manually.

Chapter 6

Budget

The budget needed to construct this system is one of the most important features of this work. The most expensive items are the galvanometer mirrors but they are changed in the second assembly for the real-time endomicroscopy. The budget is divided into two different sections; one section for the price of the components and another for the staff expenses.

6.1 Price of the components

The material expenses of the confocal endomicroscopy differ from the real-time system.

6.1.1 Confocal Endomicroscopy

Table 6.1: Price of the components of the confocal endomicroscope

Component	**Price**
Fiber bundle: FIGH-30-850N, Fujikura, Koto, TKY, Japan	698.20 USD
Laser Diode Module: CPS520, Thorlabs Inc, Delaware, USA	147.45 USD
Galvanometer Mirrors: GVS012, Thorlabs Inc, Delaware, USA	2286.72 USD
Photodetector: APD130A2, Thorlabs Inc, Delaware, USA	916.20 USD
Scan Lens: LSM02, Thorlabs Inc, Delaware, USA	1202.98 USD
Realy Lens: AC254-030, Thorlabs Inc, Delaware, USA	68.12 USD
Objective: Plan Apochromat 10X, Motic, Weztlar, Germany	922 USD
Dichroic Mirror and Emission : Edmund optics, Newport, NJ, USA	625 USD
Pinhole: P25S, Thorlabs Inc, Delaware, USA	15.53 USD
30mm Cage Assembly optomechanics, Thorlabs Inc, Delaware, USA	44.26 USD
Endoscope: Optica HOPKINS, STZ 61029D, Karl Storz, Germany	1848.50 USD
Protection and examination Sheath, Karl Storz, Germany	365.62 USD
TOTAL expenses of the Confocal Endomicroscope	9140.58 USD

6.1.2 Real-Time Endomicroscopy

Table 6.2: Price of the components of the Real-time endomicroscope

Component	**Price**
Fiber bundle: FIGH-30-850N, Fujikura, Koto, TKY, Japan	698.20 USD
Laser Diode Module: CPS520, Thorlabs Inc, Delaware, USA	147.45 USD
Realy Lens: AC254-030, Thorlabs Inc, Delaware, USA	68.12 USD
Objective: Plan Apochromat 10X, Motic, Weztlar, Germany	922 USD
Dichroic Mirror and Emission : Edmund optics, Newport, NJ, USA	625 USD
Pinhole: P25S, Thorlabs Inc, Delaware, USA	15.53 USD
30mm Cage Assembly optomechanics, Thorlabs Inc, Delaware, USA	44.26 USD
Endoscope: Optica HOPKINS, STZ 61029D, Karl Storz, Germany	1848.50 USD
Protection and examination Sheath, Karl Storz, Germany	365.62 USD
High-Resolution CMOS Camera: DCC1545M, Thorlabs Inc, Delaware, USA	282.85 USD
TOTAL expenses of the Real-Time Endomicroscope	5017.53 USD

6.2 Performance Budget

It is also important to take into account the budget needed for the performance of this experiment. This budget is in common for both endomicroscopy modules. Considering that the average salary for an engineer is 23 USD/hour:

Table 6.3: Proffesional Staff

Process	**Hours**	**Price**
Software development	250h	5750 USD
System Assembly	50h	1150 USD
Results and data analysis	50	1150 USD
	TOTAL expenses on staff	8050 USD

6.3 Total Budget

The total budget results from the sum of the expenses of the components plus the staff.

Table 6.4: Total Budget for the Confocal Endomicroscopy

Process	**Price**
Total expenses on the components	9140.58 USD
Total expenses on the staff	8050 USD
TOTAL (Confocal Endomicroscopy)	17190.58 USD

Table 6.5: Total Budget for the Real-Time Endomicroscopy

Process	**Price**
Total expenses on the components	5017.53 USD
Total expenses on the staff	8050 USD
TOTAL (Real-Time Endomicroscopy)	13067.53 USD

Appendix A

LabView Programme

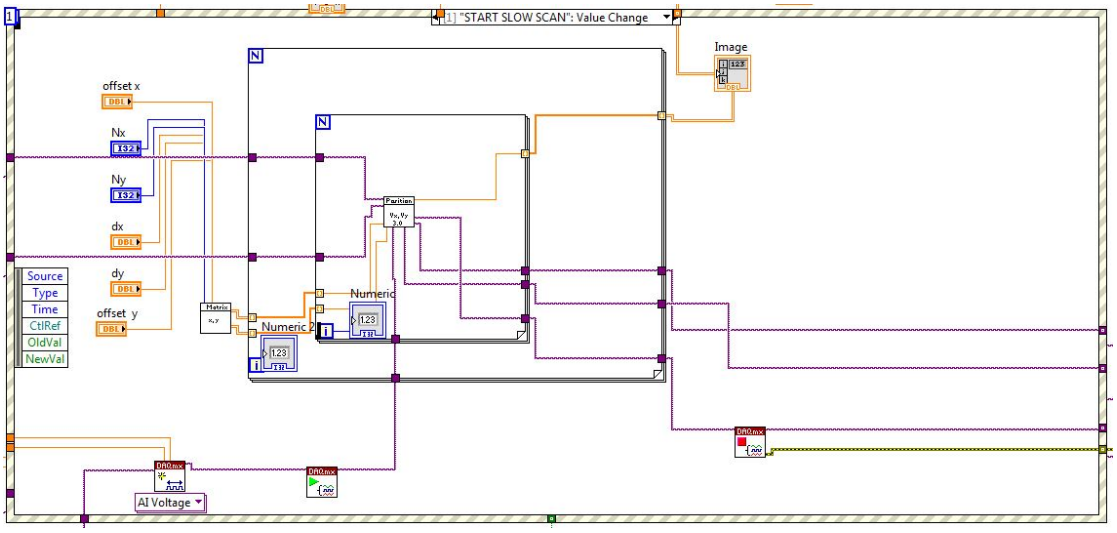

FIGURE A.1: Slow Scan Software. Main programme

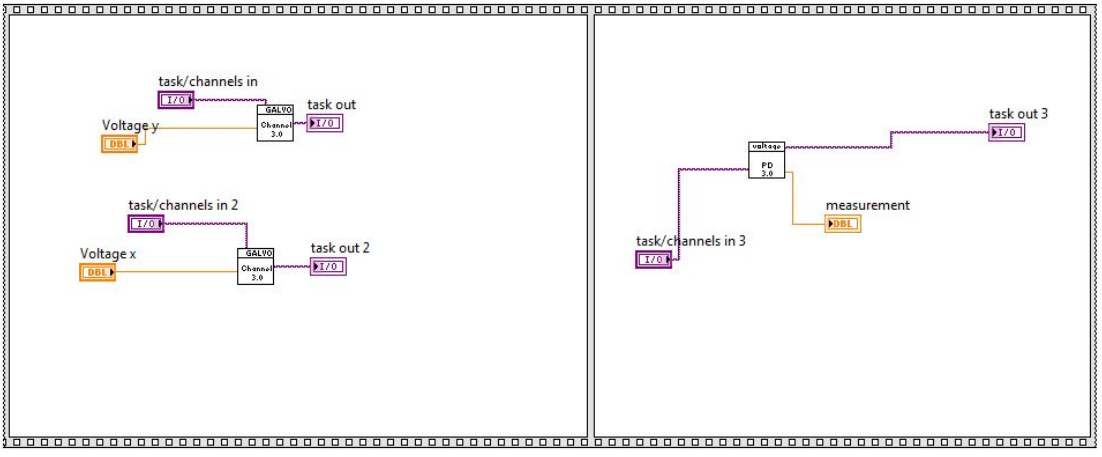

FIGURE A.2: Slow Scan Software. Position SubVI

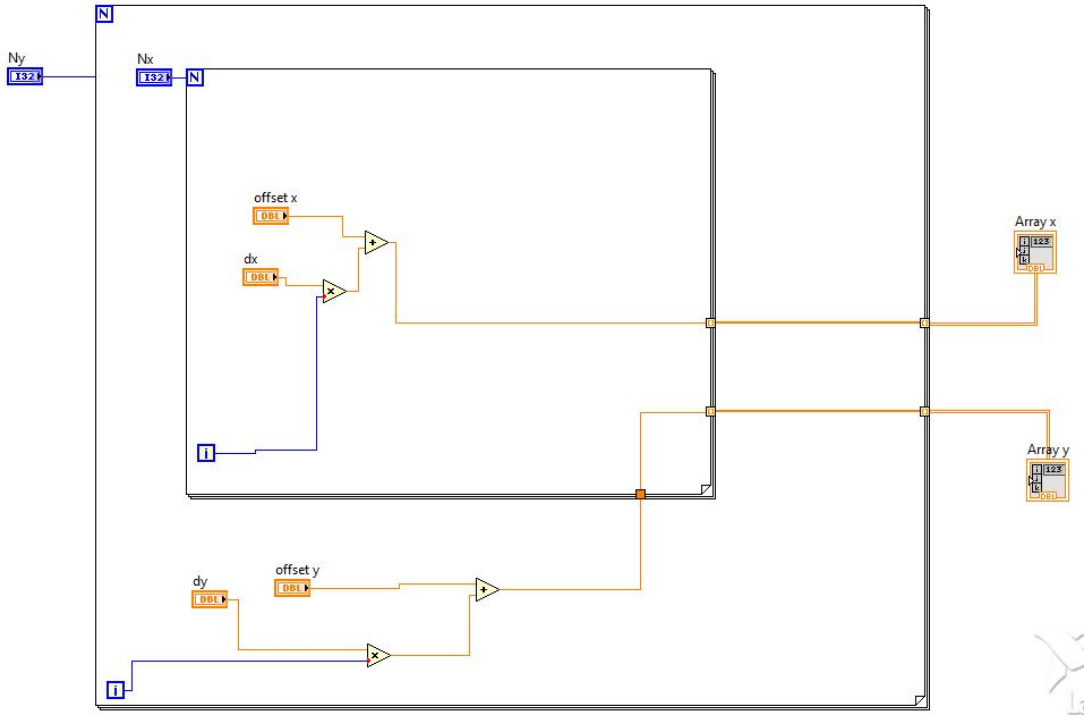

FIGURE A.3: Slow Scan Software. Matrix SubVI

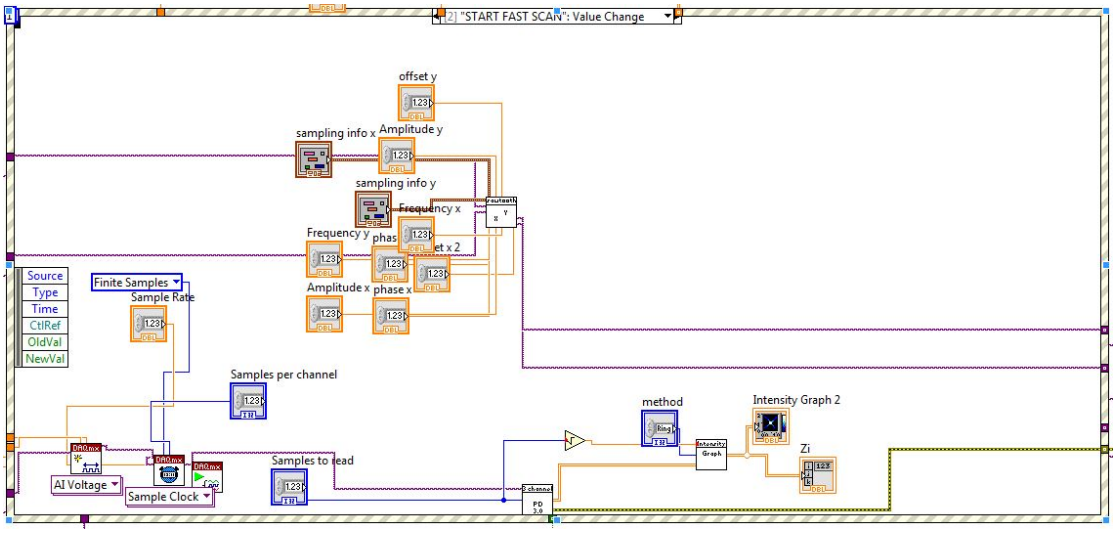

FIGURE A.4: Fast Scan Software. Main Programme

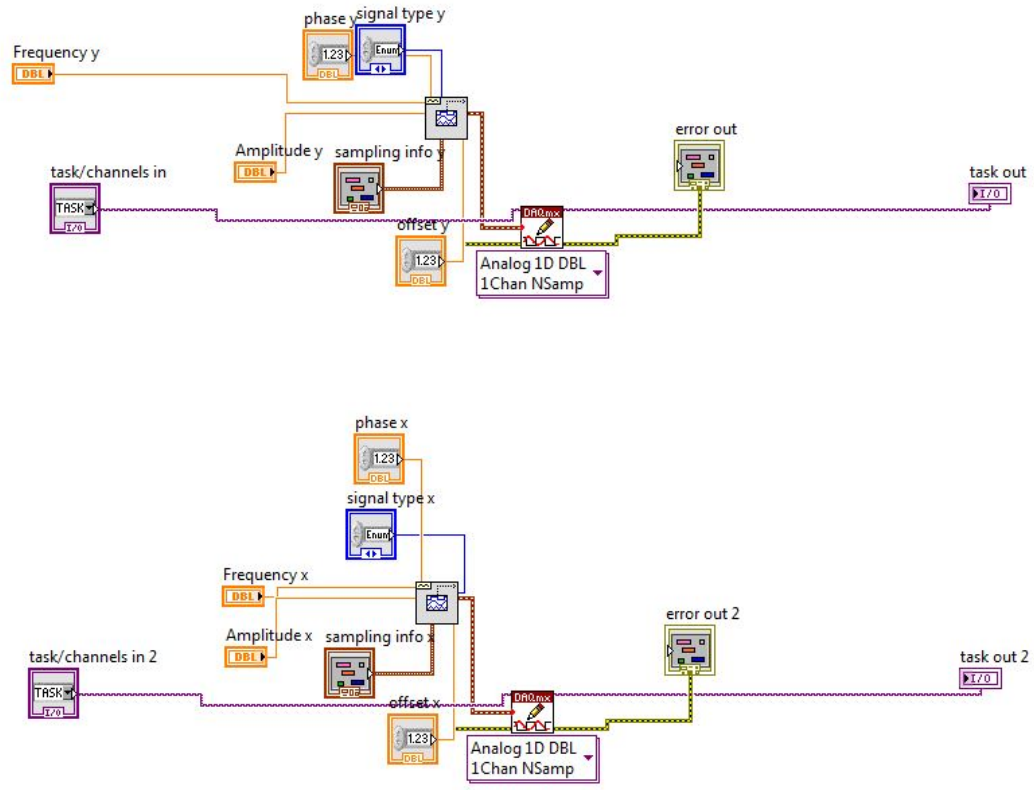

FIGURE A.5: Fast Scan Software. Sawtooth SubVI

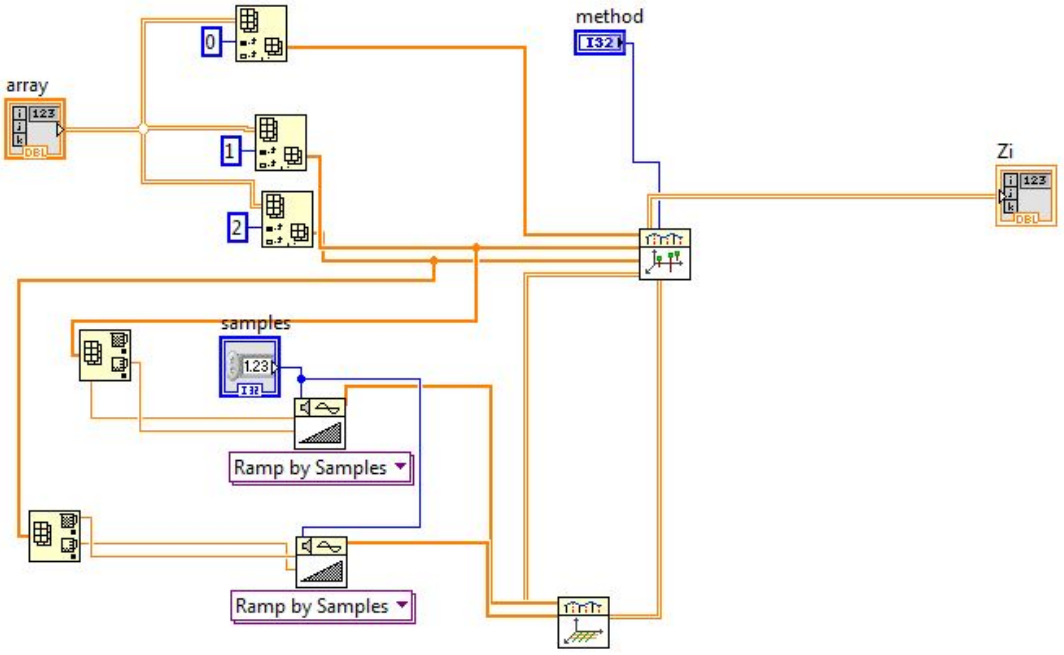

FIGURE A.6: Fast Scan Software. Interpolation SubVI

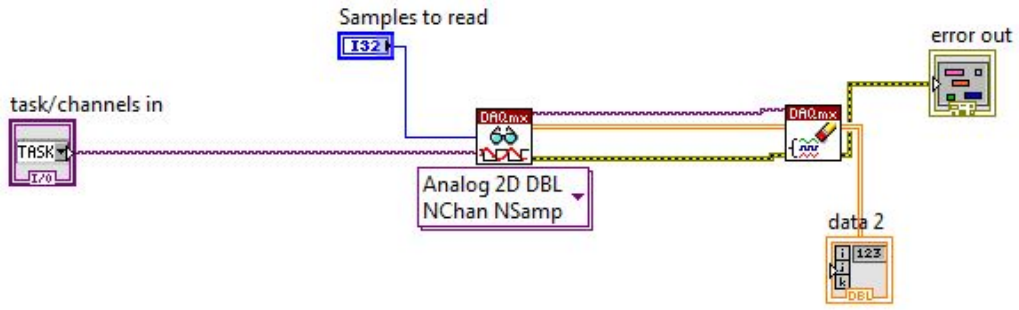

FIGURE A.7: Fast Scan Software. Different channels SubVI

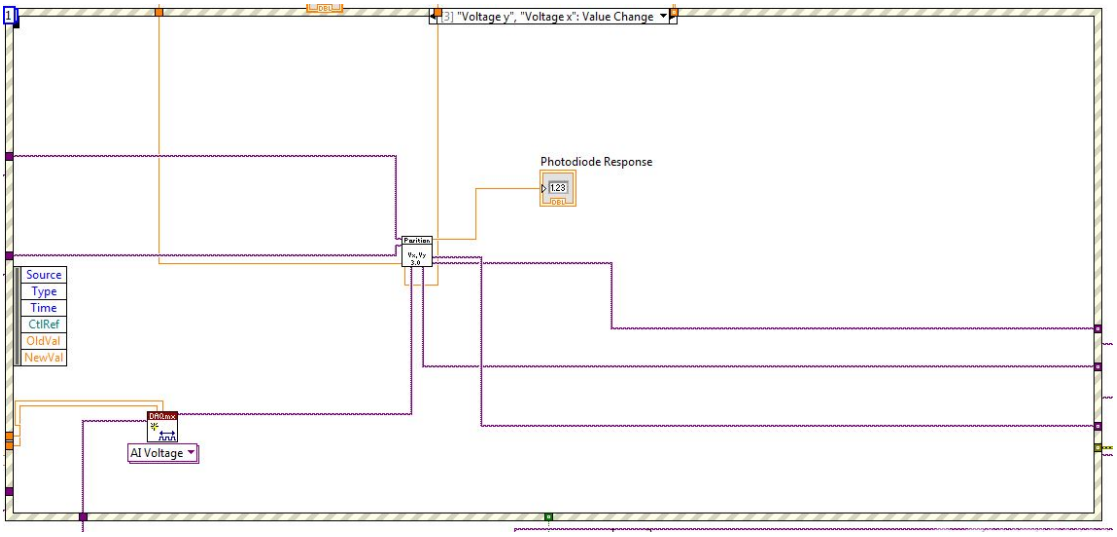

FIGURE A.8: Manual Scan Software

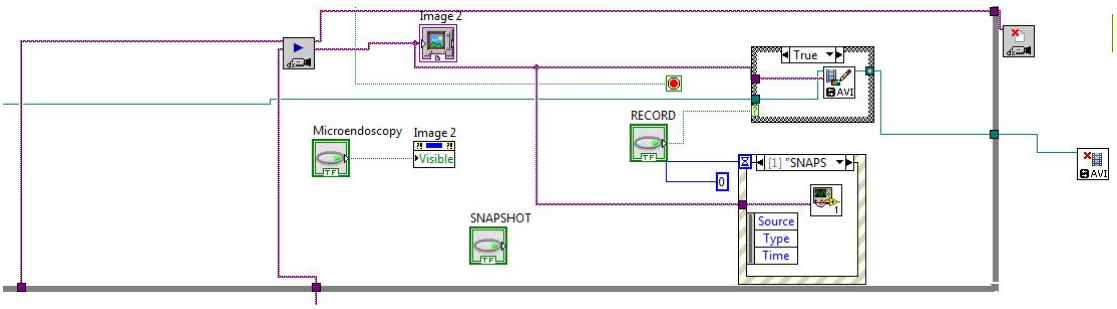

FIGURE A.9: Real-time Software

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